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Pathogenesis of Haemophilus parasuis infection in swine

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Pathogenesis of *Haemophilus parasuis* infection in swine

by

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TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

Haemophilus parasuis infection in swine is commonly referred to as Glasser's disease. Glasser described polyserositis and polyarthritis in young swine associated with a small gram negative organism in 1910.[1] Since that time, *H. parasuis* infection has been regarded as a sporadic, stress associated disease of young swine which was not considered a major cause of economic loss in swine production.[13] Following initial description of the disease, there were few reports in the scientific literature concerning the epidemiology, pathology, or pathogenesis of *H. parasuis* infection. This lack of investigation is most likely due to the fact that the disease was not considered a major cause of economic losses in swine production and the fact that the organism can be difficult to culture from experimental and field cases.

Within the last 10 years there has been renewed interest in *H. parasuis* and the role the organism plays in swine disease. One of the major factors for increased interest in *H. parasuis* infection is the fact that *H. parasuis* can produce severe disease in specific pathogen free (SPF) or other minimal disease herds.[2,5,15,21,22] The swine industry has increasingly adopted technologies which can produce minimal disease or high health status swine. Although these technologies are often effective in eliminating or decreasing losses due to major swine pathogens, they also can present unique health problems.[16] Swine within minimal disease populations often lack exposure to organisms which are normal

flora or minor pathogens in conventional swine. When these naive swine are exposed to organisms such as *H. parasuis*, severe disease often results. Another factor which has played a role in increasing the interest in *H. parasuis* infection is the emergence of Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) in North America and Europe.[8] Porcine Reproductive and Respiratory Syndrome Virus is a significant cause of respiratory disease in growing swine. Much of the morbidity and mortality in these cases is attributed to secondary bacterial pathogens such as *H. parasuis* infection.

In addition to it's role in producing systemic disease in swine, *H. parasuis* can be recovered from the nasal cavity of normal swine and is therefore considered to be part of the normal nasal flora. *H. parasuis* can also be recovered from the lungs of pigs with pneumonia. In these cases, *H. parasuis* is often isolated along with other bacterial or viral pathogens and, therefore, the role of *H. parasuis* in producing pneumonia is not clear.

Much of the experimental work related to *H. parasuis* has focused on issues such as serotypes, vaccination, and cross protection.[3,4,10,18,20] Several investigators have attempted to correlate various phenotypic features with virulence.[11,17,20] Other investigators have used experimental infection of swine and laboratory animals to determine virulence or evaluate the efficacy of bacterins.[6,7,9,12,14,19] Very little work has been done to describe the sequence of events which follows infection with *H. parasuis*. Understanding the sequence of events which follows inoculation of swine may prove useful in more fully

understanding the role of *H. parasuis* in swine disease. In addition, understanding the early events in the pathogenesis of bacterial infections may provide evidence of virulence factors.

Objectives of the Dissertation

The general goal of the dissertation research is to investigate the sequence of events which follows exposure of naive swine to *H. parasuis* in a controlled experimental infection. Accomplishing this goal will provide a better understanding of the pathogenesis of *H. parasuis* infection. This information will provide a background for drawing rational conclusions about the type of disease *H. parasuis* can produce and may suggest more effective methods of diagnosis, treatment, and prevention.

The initial goal was to develop a reproducible model of *H. parasuis* infection in swine and use that model to characterize the experimental disease using clinical, bacteriologic, and morphologic parameters. Following development of the experimental model, the objectives focused on characterizing early events in the pathogenesis of *H. parasuis* infection. Using the experimental model previously established, we sought to identify the sites of mucosal colonization, determine if colonization resulted in mucosal lesions, and determine if a specific cell type or cell structure was colonized. Initially, mucosal colonization was studied in the swine model. Subsequently, a swine nasal explant system was

developed to more fully characterize the interaction of *H. parasuis* with the nasal mucosal epithelium.

Dissertation Organization

The dissertation is presented in an alternate format with inclusion of manuscripts. The dissertation contains a general introduction, a literature review, manuscripts, and a general summary. References for each chapter are separate and immediately follow the text of that chapter. The first manuscript has been published in the Journal of Veterinary Diagnostic Investigation and describes the experimental disease. The second manuscript has been submitted to Veterinary Pathology and describes mucosal colonization in the experimental swine model. The third manuscript is prepared for submission to Veterinary Pathology and describes the development and use of the swine nasal turbinate explant system to characterize the interaction of *H. parasuis* with nasal mucosal epithelium. The Ph.D. candidate, John L. Vahle, is the first author of each paper and has been the principal investigator for each experiment.

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CHAPTER 2: GENERAL LITERATURE REVIEW

Haemophilus parasuis Infection in Swine

Haemophilus parasuis is an important cause of polyserositis and polyarthritis in growing swine.[53] Although the disease was first described by Glasser in 1910, it has received little attention from veterinary researchers.[19] Within the past 10 years there has been renewed interest in *H. parasuis* due to its role in swine disease in high health status herds and in herds infected with viral respiratory agents. This review will focus on the pertinent literature concerning *H. parasuis* infection of swine. As *H. parasuis* is thought to colonize mucosal surfaces, general mechanisms of bacterial adherence and mucosal invasion will be reviewed with particular attention to swine nasal mucosal pathogens and the human pathogen, *Haemophilus influenzae* type B (Hib).

Historical aspects

In 1910 Glasser described fibrinous serositis and polyarthritis associated with a gram negative bacterium.[19] A gram negative organism identified as *Haemophilus influenzae suis* was isolated from the respiratory tract of swine infected with Swine Influenza Virus (SIV).[37] In 1943, this bacterium was associated with polyserositis and polyarthritis and the name was shortened to *Haemophilus suis*.[24] A precise association of this bacterium with disease was difficult as it was isolated from both healthy and diseased swine. In 1976, Kilian

demonstrated that *H. suis* strains were actually *H. parasuis*.[33] Since that time *H. parasuis* has been accepted as the name of the bacterium present as normal flora and as a causative agent of disease.

Characteristics of *H. parasuis*

Haemophilus parasuis is a fastidious, small gram negative rod which varies from small singular coccobacilli to long thin chains.[53,60] The bacterium is nonhemolytic and requires V factor (NAD, nicotinamide adenine dinucleotide) for growth. Growth is supported by media containing NAD or blood agar cross streaked with *Staphylococcus spp*. Growth is usually seen within 24-48 hours.

Capsule The presence of capsule is recognized as an important virulence factor for the genus *Haemophilus*.[52] The role of capsular material in the pathogenesis of *H. parasuis* infection is not well defined. In one study capsular material was produced by 12 out of 32 strains of *H. parasuis*, however, encapsulated strains were most frequently isolated from the nasal cavities of apparently healthy swine.[45] The capsular material was reported to consist of an acidic polysaccharide. In addition, this report suggested a loss of capsular material following prolonged incubation similar to that which occurs with *Pasteurella hemolytica* serotype 1.[11] In another study, capsular material was demonstrated in a low passage isolate of a serovar which had previously been reported as unencapsulated.[61] These reports suggest that capsular expression is influenced by in vitro conditions. Detection of capsule has been based on

negative staining and agglutinability in acriflavine. Electron microscopic studies have not been reported.

Fimbriae Fimbriae are an important mechanism in bacterial adherence, however little information is available concerning the presence of fimbriae on *H. parasuis*. Fimbriae have been demonstrated by electron microscopy on *H. parasuis* inoculated on the chorioallantoic membrane of embryonated hen eggs, but were not identified on bacteria grown in conventional media.[49]

Serotype Serotype and it's relationship to virulence and immunogenicity have been the most intensely studied attribute of *H. parasuis*. The initial serologic investigation was conducted by Bakos in 1955 and identified 5 serogroups.[5] An agar gel precipitation test was developed which identified 7 serogroups.[46] The most current and complete serologic classification utilizes an immunodiffusion technique which recognizes 15 different serovars.[30] The antigen recognized in this system is thought to be a polysaccharide associated with the cell capsule or outer membrane.[30,32,71] Using this system, the most prevalent serovars in North America are (in descending order) 5, 4, 13, 14, 2, and 12 with 14% of the isolates untypeable by this system.[60] The large number of serovars and relatively large percentage of untypeable strains indicates marked heterogeneity among *H. parasuis* strains.

There have been repeated efforts to determine an association of serotype with site of isolation or virulence.[28,30,46,60,71] Results of some investigations have associated serotype 5 with isolation from systemic, rather than mucosal, sites.[29,46]

In a survey of North American isolates there was an increased frequency of serovars 4 and 5 from systemic sites; however, some of these isolates were from swine in related herds.[60] Experimental infections in swine and guinea pigs have demonstrated differences between serovars with regards to virulence with serovars 1 and 5 most consistently associated with invasiveness and virulence.[30,55,60] More recently it has been reported that there are differences in virulence among strains within a serovar.[62] These studies suggest that the heterogeneity among *H. parasuis* strains make it difficult to associate specific serotypes with virulence.

A recent study investigated the lipo-oligosaccharide of *H. parasuis*. In that study eight strains were divided in to four lipo-oligosaccharide serovars.[91] Serologic heterogeneity was demonstrated based on reactions with polyclonal and monoclonal antibodies. Similar to the immunodiffusion typing system described above, serovar based on lipo-oligosaccharide epitopes did not correlate with virulence. The lipo-oligosaccharide of *H. parasuis* appears to be similar in structure to the lipo-oligosaccharide of *H. somnus* and *H. influenzae* type B.[90,91]

Surface proteins An association between either whole cell or outer membrane proteins and virulence also remains to be defined. Morozumi and Nicolet described two sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) patterns of whole cell lysates among a limited number of strains and suggested SDS-PAGE Type II may be associated with isolation from systemic sites.[45] Rosner *et al* reported no correlation between SDS-PAGE pattern of whole

cell proteins and site of isolation.[71] Rapp *et al* examined SDS-PAGE patterns of outer membrane proteins and determined there was marked heterogeneity among *H. parasuis* isolates.[59] In addition, more than one strain of *H. parasuis* can be isolated from an individual pig.[59]

Genotype Genotypic heterogeneity has been demonstrated using restriction endonuclease fingerprinting (REF).[76] Strains with different REF profiles were often isolated from a single herd and in some cases a single pig.[76] This supports previous conclusions that more than one strain of *H. parasuis* may exist within an individual pig. None of the REF profiles of *H. parasuis* isolated from pigs with clinical disease were found in healthy swine. This may be due to the fact that inadequate numbers of isolates were examined or that isolates which produce disease are distinct from those which are part of normal flora.[76]

Natural infection

Haemophilus parasuis infection is limited to swine and is present worldwide.[53] The organism may be present as normal nasal flora or associated with a variety of disease syndromes.

Normal nasal flora *Haemophilus parasuis* is commonly isolated from the nasal cavities of healthy swine,[22,43,60,76] and is rarely isolated from the lungs of normal pigs.[53] This suggests that the organism is a normal inhabitant of the nasal cavity of swine. Epidemiologic investigations to assess the prevalence of *H. parasuis* infection based on serology have not been reported.

Polyserositis and polyarthritis syndrome Porcine polyserositis and polyarthritis due to *H. parasuis* infection is classically referred to as Glasser's disease and is the most widely recognized disease syndrome caused by *H. parasuis*. In conventional swine herds, this is typically a disease of young pigs in the postweaning period.[53] In these situations it is sporadic, has variable morbidity, and is often associated with stressors such as transportation, weaning, mixing populations of pigs, dietary changes, or poor ventilation. Mortality may approach 50%. The clinical syndrome is typically acute and includes anorexia, reluctance to move, and lameness. Upon physical examination pigs are often febrile with rectal temperatures up to 107.0 F and one or more joints may be warm, swollen, and painful on palpation. Occasionally pigs will demonstrate clinical signs of meningitis including incoordination, muscular tremors, and lateral recumbency. Pigs which survive the acute disease have rough hair coats, poor weight gains, and chronic lameness. Necropsy findings are characterized by variable amounts of fibrinous to fibrinopurulent exudate present at multiple serosal surfaces including the pericardium, pleural cavity, peritoneum, joint, and meninges.[39,53] Microscopically, these inflammatory lesions consist of fibrin, neutrophils, and occasional macrophages.[23,53]

H. parasuis infection in naive swine In Specific Pathogen Free (SPF) or other minimal disease herds, *H. parasuis* can produce severe disease.[25,39,56,73,74] In these herds, swine of all ages may be affected. Morbidity varies between 15 and 90% and mortality may be as high as 75%.[39,54,56] The clinical syndrome in

these herds can be typical of Glasser's disease,[39] however, there are often atypical disease manifestations such as septicemia or myositis.[25] Cases of septicemia often manifest as a peracute disease with high fever and diffuse to patchy erythema. Necropsy findings in these pigs may include an enlarged and congested liver and spleen, gastrointestinal mucosal hemorrhage and congestion, epicardial petechiae and ecchymoses, and increased amounts of pericardial, pleural, peritoneal, and synovial fluid.[54,70] Microscopic findings reported in one outbreak included thrombotic lesions in the glomerulus, brain, and dermal papillae.[70]

Infection in these naive herds may be introduced in one of two ways. Addition of naive breeding swine to a minimal disease herd infected with *H. parasuis* will result in severe disease in the naive swine. Additionally, introduction of apparently healthy swine infected with *H. parasuis* into naive herds has resulted in severe disease with significant economic losses in all age groups.[39,54] Strategies are being designed to detect and protect naive populations from *H. parasuis* infection.[40,41,62,63,73,77]

Interactions with other swine pathogens Although *H. parasuis* is a primary cause of polyserositis and polyarthritis, particularly in naive or stressed swine, it is often isolated in association with other swine pathogens. *Haemophilus parasuis* can be isolated from the lungs of healthy pigs and from pneumonic lungs.[60] However, the role of *H. parasuis* in pneumonia is not clear as it is often isolated in association with other pathogens such as *Mycoplasma*

hyopneumoniae, *Pasteurella multocida*, or Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). There is anecdotal evidence that disease due to *H. parasuis* infection is more common or severe in herds infected with PRRSV.[42] Little information is available from epidemiologic investigations or experimental infections to document interactions of *H. parasuis* with other swine pathogens. Two studies of experimental infections in swine have failed to demonstrate an interaction or synergism between *H. parasuis* and PRRSV.[10,78] Failure of these studies to demonstrate interactions between PRRSV and *H. parasuis* may be due to utilization of avirulent strains, inappropriate timing of PRRSV and *H. parasuis* infections, or inappropriate routes of inoculation. An interaction between PRRSV and *Streptococcus suis* has been demonstrated.[18] In a single study, pigs infected with pseudorabies virus (PRV) were accidentally exposed to *H. parasuis*. This resulted in a suppurative bronchopneumonia and *H. parasuis* and PRV antigens were detected in the lesion by immunohistochemistry.[50] Similar interactions with other viral respiratory pathogens have not been reported.

Experimental infection

Experimental models of *H. parasuis* infection have utilized a variety of inoculation routes in both swine and laboratory animals. These models have most often been used to evaluate bacterins and compare differences in virulence between strains.

Attempts to infect laboratory animals with *H. parasuis* have been limited. A study which used intraperitoneal inoculation of mice and guinea pigs determined that mice did not develop lesions and *H. parasuis* could not be consistently recovered.[44] In contrast, guinea pigs developed varying amounts of polyserositis and meningitis and *H. parasuis* was recovered from multiple tissue sites. This work led to a study utilizing intratracheal inoculation of guinea pigs to evaluate differences in virulence between strains of *H. parasuis*.[61] In this study guinea pigs inoculated with strains from serovars 1 and 5 developed a suppurative bronchopneumonia, pericarditis, and pleuritis. Although these studies have suggested that the guinea pig may be a suitable model for studying the pathogenesis of *H. parasuis* infection, additional studies using this model have not been reported.

Experimental *H. parasuis* infections have been reported in conventional, specific pathogen free (SPF), cesarean derived, colostrum deprived (CDCD), and unknown health status pigs. Intraperitoneal, intranasal, aerosol, and intratracheal routes of infection have been used in attempts to reproduce disease caused by *H. parasuis*. Several of these exposures have resulted in varying degrees of polyserositis which resembles natural *H. parasuis* infection of conventional swine. Amano *et al* reproduced polyserositis, meningitis, and septicemia utilizing an intranasal route of infection, however the type of pig used was not specified.[3] Nielsen also utilized an intranasal route of inoculation and reported typical polyserositis lesions in SPF swine.[55] Other reports which briefly describe

polyserositis lesions include aerosol and intratracheal inoculation of SPF swine.[40,41,51]

Although *H. parasuis* is most often associated with polyserositis in swine, *H. parasuis* is often recovered from the lungs of pigs with bronchopneumonia.[60] Experimental infections of swine with *H. parasuis* have only occasionally produced bronchopneumonia. Riley *et al* produced septicemic lesions and bronchopneumonia in conventional swine.[70] Other studies demonstrated that intratracheal inoculation of SPF swine with a high volume, high concentration (10^9 colony forming units (CFU) per ml or greater) inoculum led to lesions of fibrinous to purulent bronchopneumonia.[6,58] In these studies, clinical signs and lesions of polyserositis and polyarthritis typical of field infections of *H. parasuis* were not seen.

Diagnosis, treatment, and prevention

The diagnosis of *H. parasuis* in swine at necropsy is dependent upon isolation of bacteria from systemic sites in an animal with consistent morphologic lesions. Gross and microscopic lesions suggestive of *H. parasuis* include any combination of fibrinous to fibrinopurulent arthritis, meningitis, pleuritis, pericarditis, and peritonitis. Less common manifestations of *H. parasuis* infection include septicemia and myositis. Differential diagnoses for polyserositis in swine include infection with *H. parasuis*, *Streptococcus suis*, *Mycoplasma hyorhinis*, or *Escherichia coli*. Definitive diagnosis relies upon isolation of the causative

bacteria. Although *S. suis* and *E. coli* are routinely isolated in private practice and diagnostic laboratories, *H. parasuis* is often difficult to recover.[53] Additional diagnostic methods for necropsy specimens such as immunohistochemistry, *in situ* hybridization, and polymerase chain reaction (PCR) methods have not been reported. Enzyme linked immunoassay (ELISA) and immunofluorescence methods to detect antibodies to *H. parasuis* in serum have been reported.[38,77] Serologic diagnosis using the ELISA technique is available from a limited number of private diagnostic laboratories, but is not in widespread use. Better documented serologic tests may be useful in determining the status of *H. parasuis* infection within herds and obtaining epidemiologic data.

Therapy of *H. parasuis* infection has received little attention in the scientific literature. Early parenteral treatment with penicillin, ampicillin, ceftiofur, and tetracylines is often recommended.[53] If the morbidity rate within a group is high, all the pigs may receive a parenteral injection. The use of antibiotics such as tetracylines in the feed is often suggested, but there is little documentation of the benefits of feed grade medication in the treatment or prevention of *H. parasuis* infection.

Attempts to prevent disease due to *H. parasuis* can include reduction of stressors, good air quality, segregation of infected and naive pigs, and the use of bacterins. Much of the experimental literature has focused on the use of bacterins to prevent disease caused by *H. parasuis* and the ability of the bacterins to provide cross protection from nonhomologous strains. The first report is from

1975 in which an autogenous bacterin was reported to be successful in minimizing losses in an outbreak of *H. parasuis* in SPF swine.[56] This report was followed by a 1981 study which reported the widespread use of a single strain bacterin in SPF herds in Denmark.[69] In that report the authors attributed efficacy of the bacterin to either the widespread incidence of only one serovar or the fact that the protective immunogens in the bacterin were cross protective for all strains. In 1989 Smart *et al* reported the ability of a whole cell *H. parasuis* bacterin to protect against a homologous challenge in a controlled experimental setting.[75] This work was followed by several studies which have examined the ability of bacterins to protect against homologous and heterologous challenges.[31,35,40,41,63] The initial reports suggested that cross protection did occur for some strains and that virulence may correlate with immunogenicity.[41] Other investigators examined a larger number of serovars and determined that there was variability in the ability of serovars to induce protective immunity.[31] This observation has been supported by the findings of Kocur *et al* and Rapp-Gabrielsen *et al*.[35,62,63] In addition there appear to be differences in the protective immunity of strains within a serovar.[35] The marked variability in immunogenicity indicates that the protective immunogens are complex and further efforts will be required to identify protective immunogens.

Bacterial Colonization

Colonization of a mucosal surface is an important step in the pathogenesis of many bacterial infections. Colonization allows for the establishment of infection,

provides a source for transmission to other individuals, and serves as a possible site for mucosal invasion preceding systemic disease.[4,13,79] This section of the review will give a brief overview of mechanisms of colonization for selected human pathogens which have been well studied and review mucosal colonization of important swine respiratory pathogens.

General mechanisms of mucosal colonization

The process of colonization involves the establishment of a stable population of bacteria in a location.[4] An initial event in colonization may be the association of bacteria with the mucosal surface. Association has been defined as a loose, reversible attachment of bacteria in proximity to a mucosal surface.[4] This process may be enhanced by chemotaxis and motility of the bacteria and allow association with a mucous layer. In contrast to association, bacterial adhesion is characterized by stable, irreversible attachment of bacteria to a mucosal surface. This process often involves bacterial adhesins which interact with complementary receptors on the mucosal surface.

A variety of adhesins have been recognized. Fimbrial adhesins are recognized in many bacterial species and consist of filamentous structures which radiate from the bacterial surface.[4,13] Best characterized are the fimbrial adhesins of *Escherichia coli* which are important in the pathogenesis of enteric infections of humans and domestic animals.[34] Nonfimbrial proteinaceous adhesins are recognized in a variety of bacteria including *Bordetella pertussis*, *Neisseria*

gonorrhea, *Neisseria meningitis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*.[4,13,82,83] These protein structures are often outer membrane proteins. An additional type of adhesin is carbohydrate or lipid in composition. An example of this type of adhesin is the bacterial glycocalyx which may enable bacteria to remain firmly attached to epithelial cells and provide protection from phagocytes, complement, and antibiotics.[12] Although these types of adhesins are often listed in separate classes, bacteria often employ multiple mechanisms to provide a stable adherence to the mucosa. For example, *N. gonorrhea* utilizes both fimbriae and outer membrane proteins.[82] *Bordetella pertussis* utilizes two nonfimbrial secreted proteins; a filamentous hemagglutinin and the pertussis toxin.[13,83] In addition to functioning as an adhesin, the pertussis toxin is an ADP ribosylating toxin which causes increased intracellular cyclic AMP and subsequent cellular dysfunction.

Following colonization of the respiratory epithelium, many pathogens produce localized damage to cilia. Several human pathogens have been demonstrated to release ciliotoxins including *B. pertussis*, *S. pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aueriginosa*.[13] These products can produce ciliary dyskinesia, ciliostasis, and ciliotoxicity.

While pathogens such as *B. pertussis* are noninvasive and remain on the mucosal surface, many pathogens progress from mucosal colonization to invasion and systemic disease. With respect to infections of the human respiratory mucosa, two of the best characterized pathogens are *Haemophilus influenza* type

b (Hib) and *N. meningitis*. Hib is the leading cause of meningitis in the United States and can also cause epiglottitis, sinusitis, pneumonia, and otitis.[65,79] *Neisseria meningitis* is the second leading cause of bacterial meningitis in the United States.[79,81] Although these organisms produce similar types of systemic disease following nasal mucosal invasion, they utilize different mechanisms of invasion. Both pathogens attach to nonciliated epithelial cells, cause loss of ciliary activity, and sloughing of epithelial cells. *N. meningitis* invades the mucosa by entering nonciliated cells via endocytosis.[79,80] In contrast, Hib initially colonizes the mucous layer and following disruption of cellular junctions invades between epithelial cells.[16,65,79] Following epithelial invasion, both pathogens can be found in the subepithelium adjacent to lymphoid follicles.[79] The role of adhesins appears to differ between these two pathogens. Fimbriae play an important role in adhesion of *N. meningitis*, while Hib appears to employ both fimbrial and nonfimbrial adhesins.[79,84,85]

As *H. influenzae* type B is in the same genus as *H. parasuis*, it is useful to more fully explore the pathogenic mechanisms of Hib infections in humans. Capsulate strains of *H. influenzae* associate with a gel like matrix at the apical surface of nasal mucosal epithelial cells, while nonencapsulated strains can directly adhere to epithelial cells.[64] Other workers have demonstrated that Hib initially associates with a mucus layer and specifically adheres to sugar residues within the mucus.[17,65,88] Hib has been shown to decrease ciliary beat frequency and produce ciliostasis.[17] The mechanism of ciliostasis may be mediated by

either lipopolysaccharide (LPS) or a low molecular weight substance which has not been fully characterized.[47,48] In addition, there is evidence that Hib can increase mucus secretion from epithelial cells.[47,65] It is suggested that increased mucus can lead to partitioning of the mucus layer with the outer layer remaining stationary and thereby allow bacteria to remain within the nasal cavity. The bulk of experimental evidence suggests that Hib produces epithelial cell degeneration and then attaches to nonciliated epithelial cells.[17,65,79] Fimbriae which attach to sialic acid containing lactosylceramide residues appear to be involved in attachment to nonciliated epithelial cells.[84,85] The fimbrial subunit gene is highly conserved among Hib strains and is related to the gene encoding *E. coli* fimbriae.[85] Hib appears to invade the mucosa in areas of epithelial cell damage and can be detected within the submucosa.[65,149] Little is known about the mechanisms responsible for mucosal invasion or translocation into the blood stream. In a rat model, Hib infection produces an early bacteremia.[72] Hib has been shown to replicate to high numbers within the blood stream[48] suggesting that an extravascular systemic site of replication is not necessary prior to localization within the meninges.

Mucosal colonization of selected swine respiratory pathogens

The nasal cavity and oropharynx are important sites of localization for a variety of swine pathogens involved in progressive atrophic rhinitis, pneumonia, and systemic disease. In a survey of Iowa swine in 1967, *Bordetella*

bronchiseptica, *Pasteurella multocida*, *Haemophilus spp.*, *Streptococcus spp.*, and *Mycoplasma hyorhinis* were among the organisms recovered.[22] A more recent survey of slaughter swine which evaluated the NAD dependent *Pasteurellaceae* found that *H. parasuis* is a common inhabitant of the nasal cavity and *Actinobacillus pleuropneumoniae* is present in the retropharyngeal tonsil and, to a lesser extent, the nasal cavity.[43] The tonsil is also an important site of colonization for pathogens such as *Streptococcus suis* and *P multocida*.[1,2,87]

Due to their role in progressive atrophic rhinitis, *P. multocida* and *B. bronchiseptica* have been the most extensively studied pathogens of the swine nasal cavity. Toxigenic strains of *P. multocida* type D are commonly implicated in progressive atrophic rhinitis and *P. multocida* type A strains are more commonly implicated in subacute to chronic pneumonia, often in association with other respiratory pathogens. *Pasteurella multocida* has been shown to colonize the tonsil and nasal cavity of conventional and gnotobiotic swine.[1,2,20,57] In studies using quantitative methods, the tonsil was colonized more extensively than the nasal cavity and was thought to be the primary site of colonization.[1,2,57] Colonization of *P. multocida* has also been studied using *in vitro* systems.[9,14,27,36,57] One study failed to demonstrate adherence of *P. multocida* to isolated swine nasal epithelial cells.[9] In the same study *B. bronchiseptica* was readily adherent to nasal epithelial cells. Other investigators have demonstrated colonization of nasal turbinate fragments and tracheal ring explants.[14,27,57] In one of these studies adherence of *P. multocida* to tracheal ring mucosa was markedly enhanced by

preinfection with *B. bronchiseptica.*[14] Bacteria initially contact the mucous layer of a mucosal surface and *P. multocida* has been shown to colonize swine mucous preparations.[27,36] The cellular mechanisms involved in adherence of *P. multocida* are not known. It has been shown that the presence of a capsule is negatively correlated with adherence to tracheal ring mucosa and mucus.[27] Fimbrial expression has been correlated with virulence and may play a role in adhesion.[8]

Bordetella bronchiseptica is most important as a co-pathogen with *P. multocida* in progressive atrophic rhinitis. *B. bronchiseptica* can also be the sole cause of mild rhinitis or hemorrhagic pneumonia in young pigs. Similar to other *Bordetella spp.*, the organism preferentially colonizes ciliated epithelial cells in the nasal cavity.[89] Using in vitro systems, *B. bronchiseptica* has been shown to adhere to isolated swine nasal epithelial cells and tracheal ring explants.[9,15,26] Mechanisms of adherence for *B. bronchiseptica* have been shown to include hydrophobicity and fimbrial adhesins.[7,26] There is recent evidence that attachment of *B. bronchiseptica* to porcine neutrophils is mediated by integrin and carbohydrate dependant mechanisms.[68]

Streptococcus suis is an important pathogen of swine and can produce septicemia, meningitis, polyserositis, polyarthritis, purulent bronchopneumonia, and necrohemorrhagic pneumonia.[66,67] Experimental evidence suggests that the palatine tonsil is the primary site of mucosal colonization.[87] Although little is known about the specific mechanisms of adhesion, there is evidence that capsule may play a role as capsular thickness was positively correlated with adherence of

S. suis to lung slices.[21] The same study determined that hydrophobicity was not correlated with adherence. Although there is little known about virulence factors specifically involved in adherence, there is evidence that two proteins, muramidase-related protein (MRP) and extra-cellular factor (EF) are associated with virulence.[86] The function of these virulence factors remains to be defined.

In contrast to other swine respiratory mucosal pathogens, there is little known about the colonization of *H. parasuis*. *Haemophilus parasuis* can be isolated from the nasal cavity of healthy swine and swine with rhinitis.[20,60] In a recent survey involving NAD dependant *Pasteurelleacae* recovered from the tonsil, nasal cavity, or lungs of slaughter swine, *H. parasuis* was consistently isolated from the nasal cavity and isolated from the tonsil of only one pig.[43] Researchers conducting infectivity studies with *H. parasuis* have not often described recovery from mucosal sites. A single study reported *H. parasuis* was recovered from nasal secretions and not from the tonsil; however, in this same report antigens of *H. parasuis* were not identified in the nasal cavity but were present in the tonsil.[3]

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CHAPTER 3. EXPERIMENTAL REPRODUCTION OF *HAEMOPHILUS PARASUIS* INFECTION IN SWINE: CLINICAL, BACTERIOLOGIC, AND MORPHOLOGIC FINDINGS.

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ABSTRACT. *Haemophilus parasuis* is a common cause of polyserositis and polyarthritis in swine. Little is known about the mucosal and systemic sites of replication and lesions following aerosol exposure to *H. parasuis*. In this experiment, 5 week old cesarean derived, colostrum deprived (CDCD) pigs were inoculated intranasally with an inoculum containing 2 X 10^9 colony forming units of *H. parasuis*. Two principals and one control pig were necropsied at 12, 36, 84, or 108 hours postinoculation (PI) and samples obtained for bacteriologic culture and microscopic examination. Inoculated pigs developed clinical signs of inappetence, reluctance to move, lameness, and a serous nasal discharge. Macroscopic findings included a fibrinous polyserositis and polyarthritis 36 hours PI which became progressively more severe at 84 and 108 hours PI. No lung lesions were grossly visible. Microscopic lesions included a mild purulent rhinitis at each post inoculation interval and fibrinous to fibrinopurulent synovitis and serositis at 36,

84, and 108 hours PI. A focal suppurative bronchopneumonia was observed in one pig examined at 36 hours PI. The nasal cavity and trachea were the only mucosal sites from which *H. parasuis* was reisolated. *H. parasuis* was isolated from the blood and systemic sites at 36, 84, and 108 hours PI. Findings presented indicate that intranasal inoculation of 5 week old CDCD pigs with *H. parasuis* results in clinical signs and lesions of polyserositis and polyarthritis typical of field cases and is a useful model for the study of *H. parasuis* pathogenesis. The results also suggest that *H. parasuis* initially colonizes the nasal mucosa.

INTRODUCTION

Haemophilus parasuis is a common cause of polyserositis and arthritis in swine and is often referred to as Glasser's disease.[12] It is most often considered a sporadic, stress associated disease of young pigs in conventional swine herds. However, in naive herds, *H. parasuis* can result in severe disease with high morbidity and mortality in swine of all ages.[5,14] Disease caused by *H. parasuis* is also important in herds infected with Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), and *H. parasuis* has been isolated with increased frequency in the upper midwest since the appearance of PRRSV.[8]

In addition to its role as a causative agent of polyserositis, *H. parasuis* can be isolated from the nasal secretions of apparently healthy pigs and pigs with rhinitis or mucous nasal discharge.[3,15] Additionally, *H. parasuis* is often isolated

from the lungs of pigs with pneumonia,[4,15] however the role of *H. parasuis* in respiratory disease is unclear as other bacterial or viral pathogens are often identified in association with *H. parasuis.*

H. parasuis infection has been experimentally induced by intraperitoneal[10] or intranasal[1] inoculation of conventional swine and by inoculation of specific pathogen free (SPF) or cesarean derived, colostrum deprived (CDCD) pigs by intratracheal,[11] aerosol,[6,7,18] or intranasal[13] routes. Polyarthritis and polyserositis, typical of natural *H. parasuis* infection, were produced by these methods. Intranasal inoculation of conventional pigs with a high dose of *H. parasuis* resulted in a fibrinosuppurative bronchopneumonia, but not typical polyserositis and polyarthritis.[1]

Although there are prior reports of experimental reproduction of *H. parasuis* infection in both conventional and naive swine, little in known about initial sites of replication or the sequence of events which follows a respiratory challenge of *H. parasuis*. The purpose of this investigation was to describe the clinical, bacteriologic, and morphologic findings at various time points following intranasal challenge of cesarean derived, colostrum deprived pigs with *H. parasuis.*

MATERIALS AND METHODS

Experimental animals - Twelve cesarean derived, colostrum deprived pigs were raised in isolation and purchased from a commercial source at 5 weeks of age. Upon arrival at the isolation facility a single nasal swab was obtained from

each pig and was negative for *H. parasuis* following inoculation on blood agar cross streaked with *Staphylococcus epidermidis*. Pigs were housed in isolation rooms with concrete floors and automatic ventilation, fed an 18% corn-soybean meal ration, and provided water ad libitum.

Challenge strain - *H. parasuis* was isolated from the pericardium of a pig submitted to the Iowa State University Veterinary Diagnostic Laboratory with lesions of severe polyserositis, polyarthritis, and meningitis and designated strain HPS 5. To minimize the effects of *in vitro* procedures on virulence, the isolate was passed twice *in vitro* and stored at - 70 C.

Challenge preparation - The *H. parasuis* isolate was thawed at room temperature, grown overnight in supplemented M96 broth, and inoculated onto supplemented pleuropneumonia-like organism (PPLO) agar as described previously.[16] Plates were incubated at 37 C for 18 to 24 hours. Bacteria from each plate were harvested in 5.0 ml cold, sterile, phosphate buffered saline solution (PBSS), ph 7.2. The bacterial suspension was centrifuged (5 minutes at 3000 g), the supernatant discarded, and bacterial cells resuspended in 4.0 ml PBSS. Standard plate count of the challenge inoculum was 2.2×10^9 colony forming units (CFU) per ml.

Experimental design - Control and principal groups were housed separately in isolation rooms. Eight experimental pigs were inoculated with 0.5 ml of the challenge inoculum in each nostril to give a total inoculum dose of approximately 2×10^9 CFU. Four control pigs were inoculated with 0.5 ml of sterile PBSS in

each nostril. Clinical signs were monitored and rectal temperatures were obtained and recorded three times daily. Two pigs from the principal group and one pig from the control group were necropsied at 12, 36, 84, and 108 hours post inoculation. Gross lesions were recorded and samples obtained for bacterial isolation and microscopic examination.

Bacterial isolation - Specimens for bacterial culture included blood, tonsil, lung, liver, spleen, and swabs from the nasal cavity, trachea, pericardium, pleura, peritoneum, meninges, and synovia. Specimens were cultured by direct inoculation of both blood agar cross-streaked with *Staphylococcus epidermidis* and tubes of supplemented M96 broth.[16] Broth cultures were incubated overnight at 37 C, then inoculated onto blood agar plates. Blood agar and M96 broth were examined after 24 and 48 hours incubation at 37 C. Representative colonies of bacteria isolated from each site were identified biochemically as *H. parasuis* and submitted to the Iowa State University Veterinary Diagnostic Laboratory for confirmation.

Microscopic examination - Nasal turbinates, tonsil, trachea, lung, brain, liver, spleen, lymph node, heart, joint capsule, and any tissues with adherent exudate were collected in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin (HE). Histologic lesions were evaluated by light microscopy.

RESULTS

All control pigs remained clinically normal throughout the experiment and did not have lesions or positive bacterial cultures at necropsy.

Clinical evaluation. The 2 pigs euthanized and examined at 12 hours PI did not have clinical signs or elevations in rectal temperatures. At 16 hours PI 5 of 6 inoculated pigs were slightly lethargic and had mild elevations in rectal temperature. By 36 hours PI 2 of 6 pigs were reluctant to move and had rectal temperatures of 41.6 C. When forced to move they were non-weight bearing on one limb. Remaining pigs (4/4) developed similar elevations in body temperature at 60 to 70 hours PI and additional clinical observations included swollen joints, mucopurulent nasal discharge, and lateral recumbency. At 84 hours PI 1 of 4 pigs was found dead and at 108 hours PI 1 of 2 pigs was found dead.

Macroscopic findings. In pigs euthanized and necropsied at 12 hours PI, gross lesions were mild and consisted of moderate amounts of cloudy, straw colored fluid within the pleural, pericardial, and peritoneal cavities. At 36 hours PI fibrin clots were present in the pericardial, pleural, and peritoneal fluid, and the hock, carpal, and stifle joints were distended by variable amounts of fibrinopurulent exudate. Pigs examined at 96 and 108 hours PI had large amounts of partially organized fibrinopurulent exudate within the pericardial, pleural, and peritoneal cavities. This exudate often covered the serosal surface and was focally adherent to the abdominal organs. In 1 male pig examined at 96 hours PI and 1 male pig examined at 108 hours PI, there was extension of the

exudate into the vaginal tunics producing a fibrinopurulent periorchitis. Joint lesions were most severe at 96 and 108 hours PI as the joint capsule was markedly distended by large amounts of fibrinopurulent exudate which often extended into the adjacent periarticular soft tissues and fascial planes. There was no evidence of erosion or ulceration of the articular cartilage in any of the pigs examined. The livers of all inoculated pigs examined at 36, 96, and 108 hours PI were slightly pale and mottled dark red to tan.

Microscopic findings. Microscopic lesions were limited to the nasal mucosa in pigs sampled at 12 hours PI and consisted of mild to moderate patchy infiltrates of neutrophils in the nasal submucosa and focally intense aggregates of neutrophils within the nasal mucosal epithelium (Fig. 1). Similar lesions were present at 36 (1/2 principals), 84 (1/2 principals), and 108 (2/2 principals) hours PI. Sections from both control and principal pigs contained focal loss of cilia and detachment of the nasal mucosa which were interpreted as artifacts due to processing and handling of the tissues.

Synovial lesions were present in all principals examined after 12 hours. In 1 of 2 principal pigs examined at 36 hours PI the synovium was edematous and capillaries were congested. In the other principal examined at 36 hours PI, the joint lesions consisted of fibrinopurulent synovitis. The synovial membrane surface was focally covered by exudate composed of moderate amounts of fibrin and variable numbers of neutrophils (Fig. 2). The loose connective tissue of the synovia was edematous and infiltrated by scattered neutrophils. Capillaries in the

synovia were congested and large numbers of neutrophils were adherent to the capillary endothelium. In all principal pigs examined at 84 and 108 hours PI, joint lesions were qualitatively similar to lesions present at 36 hours PI but more severe in extent and distribution (Fig. 3). An additional finding in joints examined at these later time points were increased numbers of lymphocytes in the subepithelial connective tissue of the synovia.

Inflammation was present at 36, 84, and 108 hours PI on the pleural surface of the lung and peritoneal surfaces of the liver, spleen, and small intestine. The exudate consisted of variable amounts of fibrin, numerous neutrophils, lesser numbers of macrophages, and scattered small aggregates of basophilic granular material.

Microscopic lesions in the lung were detected in one principal which was examined at 36 hours PI. In this pig, scattered interlobular septa were distended by serous exudate, moderate numbers of neutrophils, lymphocytes, and macrophages and interlobular lymphatics were dilated and contained scant eosinophilic material. In adjacent lobules, there were focally extensive areas in which alveolar lumens contained variable numbers of neutrophils, macrophages, and scant amounts of fibrin. Within these areas alveolar septal capillaries were congested, and occasional bronchioles contained mild numbers of neutrophils.

Bacteriologic findings. Results of bacterial cultures are presented in Table 1. In general, bacterial cultures were either sterile or yielded pure cultures of *H. parasuis*. At 12 hours PI, *H. parasuis* was recovered from the nasal cavity

and trachea. *Haemophilus parasuis* was isolated from blood cultures only in pigs examined at 36 hours PI. Positive isolations from systemic tissue sites were present in all pigs examined at 36, 84, and 108 hours PI, however the number of positive culture sites per pig varied from 1 to 6.

DISCUSSION

In the present study, intranasal inoculation of CDCD swine with a recent field isolate of *H. parasuis* resulted in typical clinical signs and gross and microscopic lesions of fibrinous polyserositis and polyarthritis which resemble field cases of *H. parasuis* infection. Previous reports have described experimental reproduction of *H. parasuis* infection in SPF or CDCD swine by intratracheal,[11,17] aerosol,[6,7,18] or intranasal routes.[13] These prior studies focused on virulence or immunogenic differences among *H. parasuis* serotypes or strains and do not report the mucosal and systemic sites of bacterial replication at different time points or describe the microscopic lesions following a respiratory challenge of *H. parasuis*.

Although *H. parasuis* is often isolated from the lungs of pigs with pneumonia,[4,15] other bacterial or viral pathogens are often identified. Determination of the ability of *H. parasuis* to produce pneumonia in the absence of other infectious agents would be useful. Pneumonia, as determined by gross examination, has not been a feature of previous reports of *H. parasuis* infections in CDCD swine.[6,7,17,18] This is in agreement with the present study in which

H. parasuis was not isolated from lung, none of the pigs had macroscopic evidence of pneumonia, and only 1 of 8 pigs had a small focal area of suppurative bronchopneumonia detected upon microscopic examination. A recent abstract[1] describes gross and microscopic lesions of fibrinosuppurative bronchopneumonia following intranasal inoculation of conventional pigs with a large dose of a concentrated *H. parasuis* inoculum. Typical systemic lesions of polyserositis and polyarthritis were not found in that study. The apparent discrepancy between that report and the current and other studies using SPF or CDCD swine may be due to differences in the inoculum dosage, virulence differences of the *H. parasuis* strain used, or concurrent infectious agents present in the conventional swine. The weight of experimental evidence suggests that *H. parasuis* infection by itself does not produce significant pneumonic lesions. Evaluation of additional isolates may detect strains which have a tropism for the lung and produce significant pneumonia. The ability of *H. parasuis* to produce pneumonia in swine with concurrent viral respiratory infections such as Porcine Reproductive and Respiratory Syndrome Virus, Swine Influenza Virus, or Porcine Respiratory Coronavirus remains to be determined.

Determination of the sequence of events following inoculation is useful in defining the pathogenesis of *H. parasuis* infection. A study which examined *H. parasuis* isolations from swine at slaughter detected *H. parasuis* in the nasal cavity of swine, but failed to detect *H. parasuis* from the tonsil.[9] In the present study, the earliest lesion detected was a purulent rhinitis in pigs sampled at 12

hours PI and *H. parasuis* was reisolated from the nasal cavity of these pigs. *H. parasuis* was not isolated from the tonsil in pigs at any time point. These findings suggest that *H. parasuis* may colonize the nasal cavity as the initial event in infection and the tonsil may not be important as a site of mucosal colonization.

This is the first study which has reported microscopic lesions in the nasal turbinate following experimental infection with *H. parasuis*. Purulent rhinitis was identified as early as 12 hours PI and persisted to the end of the study. The reproduction of nasal discharge and rhinitis in this report is interesting, since a previous survey of *H. parasuis* isolates reported isolation of *H. parasuis* from pigs with mucus nasal discharge or rhinitis.[15] Additionally, a report which investigated the role of *Pasteurella multocida* and *H. parasuis* in progressive atrophic rhinitis identified mild nasal turbinate atrophy in pigs naturally infected with *H. parasuis* and suggested that *H. parasuis* may play a role as an initiating factor in *P. multocida* infection and subsequent progressive atrophic rhinitis.[2] In the current report large numbers of *H. parasuis* were inoculated directly into the nasal cavity. This infectious dose is likely in excess of natural exposure, and therefore the ability of *H. parasuis* to produce significant nasal lesions under field situations is not known.

Morphologic lesions at systemic sites and reisolation of *H. parasuis* from systemic sites were first present at 36 hours PI. Blood cultures were positive only at 36 hours PI and suggest that invasion of the blood stream occurred between 12 and 36 hours PI. The spectrum of lesions at systemic sites included synovitis,

pericarditis, pleuritis, peritonitis, and, in two male pigs, periorchitis. These lesions were qualitatively similar at 36, 84, and 108 hours PI, however lesions became progressively more severe and extensive. Meningitis was not detected in this study, however *H. parasuis* was isolated from a meningeal swab of 1 pig at 84 hours PI. Meningitis is described in field cases[12] and has been a sporadic finding in experimental infections.[6]

Haemophilus parasuis was isolated less frequently from systemic sites at later time points and lesions with extensive inflammation were often negative at 84 and 108 hours PI. These findings are in agreement with a previous report in which *H. parasuis* was readily recovered from pigs in the acute stages of experimental infection, but was isolated only sporadically from pigs with lameness at later time points.[11] In addition many field cases with fibrinous polyserositis and polyarthritis are culture negative.[12] The failure to recover an organism from these cases may be due to factors such as prior antibiotic therapy, prolonged postmortem interval, or improper sample collection and handling. In some cases negative culture results may be due to the fact the organism is no longer present in the lesion or is present at very low levels. Additional diagnostic methods such as immunohistochemistry or ELISA to identify *H. parasuis* antigens in tissues or exudates would be useful in both experimental or diagnostic situations.

This report suggests that *H. parasuis* colonizes the nasal turbinates and produces a mild rhinitis as an initial event in the pathogenesis of *H. parasuis* infection of CDCD swine. Bacteremia follows with bacterial replication at multiple

serosal surfaces and ensuing lesions of fibrinosuppurative polyserositis and polyarthritis. Pneumonia and meningitis were not prominent lesions in this study. Marked phenotypic and genotypic heterogeneity has been demonstrated for *H. parasuis*[12] and *H. parasuis* serovars can differ in virulence.[16] Other strains of *H. parasuis* may produce a spectrum of lesions different than that reported in this study.

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Table 1. Bacteriologic Findings in Pigs Inoculated with *Haemophilus parasuis*

Pig	Hours Post	Results*										
		Necropsy Specimens+										
Num.	Inoc.	Bl	Na	To	Tr	Lu	Pc	Pl	Pt	Jt	Mn	Li	Sp
1	12	0	+	0	0	0	0	0	0	0	0	0	0
2	12	0	+	0	+	0	0	0	0	0	0	0	0
3	36	+	+	0	0	0	0	0	0	+	0	0	0
4	36	+	0	0	0	0	0	+	+	0	0	+	+
5	84	0	0	0	0	0	0	0	0	+	0	0	0
6	84	0	+	0	+	0	0	+	+	+	+	0	0
7	108	0	0	0	0	0	0	0	0	+	0	0	0
8	108	0	0	0	0	0	0	0	0	+	0	0	0

* + = Isolation of *Haemophilus parasuis*; 0 = Negative culture

\+ Bl = blood culture; Na = nasal turbinate swab; To = tonsil;
Tr = tracheal swab; Lu = lung; Pc = pericardial swab;
Pl = pleural swab; Pt = peritoneal swab; Jt = joint swab;
Mn = meningeal swab; Li = liver; Sp = spleen

Figure 1. Nasal turbinate, 5 week old pig, 12 hours PI. The submucosa contains increased numbers of inflammatory cells and there are focal intraepithelial aggregates of neutrophils (arrowhead). HE. Bar = 30 μm.

Figure 2. Synovium, 5 week old pig, 36 hours PI. There is a focal accumulation of fibrinopurulent exudate adherent to the synovial membrane surface. The subsynovium is edematous. HE. Bar = 50 μm.

Figure 3. Synovium, 5 week old pig, 84 hours PI. The synovial membrane surface (arrowheads) is covered by a thick layer of fibrin, neutrophils, and cellular debris. A subsynovial vessel (arrow) contains numerous adherent neutrophils. HE. Bar = 30 μm.


1
2

CHAPTER 4. INTERACTION OF *HAEMOPHILUS PARASUIS* WITH NASAL AND TRACHEAL MUCOSA FOLLOWING INTRANASAL INOCULATION OF CESAREAN DERIVED COLOSTRUM DEPRIVED (CDCD) SWINE

A paper submitted to Veterinary Pathology

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ABSTRACT. Twenty three cesarean derived, colostrum deprived pigs were obtained at 5 weeks of age and inoculated intranasally with either 1.4 X 10^8 colony forming units of *Haemophilus parasuis* or sterile phosphate buffered saline. Pigs were euthanized at 4, 8, 12, 18, 26 or 36 hours post inoculation and tissues from the oropharynx and respiratory tract were obtained for bacterial culture, for immunohistochemistry to *H. parasuis* antigens, and for light and transmission electron microscopic evaluation. *Haemophilus parasuis* was consistently isolated from the nasal cavity (17/17) and trachea (13/17) and rarely isolated from the lung (3/17) and peripheral blood (1/17). Antigens of *H. parasuis* were sporadically detected on the nasal mucosa (6/17) and trachea (8/17). Light microscopic lesions included submucosal and intraepithelial infiltrates of neutrophils and infrequent, patchy loss of cilia. Ultrastructural changes in nasal mucosal epithelial cells included cell protrusion, loss of cilia, and dilation of the cytocavitary network. Bacteria were infrequently observed and were either within an amorphous material at the apical surface of the cilia or were between individual cilia. These results suggest *H. parasuis* initially associates with the nasal and tracheal mucosa and

can induce a suppurative rhinitis with nasal mucosal epithelial cell degeneration. This process may represent an initial event in the pathogenesis of *H. parasuis* infection of swine.

INTRODUCTION

Haemophilus parasuis is a common cause of fibrinous polyserositis and polyarthritis in young swine.[24] In addition, *H. parasuis* can cause septicemia and myositis in naive swine and is often cultured from the lungs of pigs with pneumonia and the nasal cavity of conventional swine.[11,26,27,30,34] In recent years there has been increased interest in disease caused by *H. parasuis*, in part, because of the disease severity when *H. parasuis* is introduced into naive populations.[31,32] Expanded use of high-health animal husbandry technologies in the swine industry has apparently resulted in greater numbers of swine susceptible to disease caused by *H. parasuis*.

There is relatively little known about the pathogenesis of *H. parasuis*. Phenotypic features do not necessarily correlate with virulence and specific virulence factors have not been identified.[19,24,28] We recently studied the sequence of events which follow intranasal inoculation of cesarean derived, colostrum deprived (CDCD) pigs with *H. parasuis*. In that study, *H. parasuis* was consistently isolated from the nasal cavity, but was not recovered from other mucosal sites.[35] A study which examined isolates obtained from slaughter swine determined that *H. parasuis* was a common inhabitant of the nasal cavity, but was

not recovered from the tonsil.[17] These studies indicate that the nasal mucosa may be the initial site of mucosal colonization for *H. parasuis*.

Determining the sites and mechanisms of mucosal colonization is important in understanding the pathogenesis of bacterial infections and identifying virulence factors. This study was designed to determine the specific sites of mucosal colonization of *H. parasuis* in CDCD swine and determine if morphologic changes of the mucosa are associated with colonization as determined by qualitative bacterial culture, immunohistochemistry, and light and transmission electron microscopy.

MATERIALS AND METHODS

Bacterial inoculum - An isolate of *H. parasuis* recovered from the pericardium of a pig with severe polyserositis, polyarthritis, and meningitis designated HPS 5 was used in this study. The isolate was passed twice and stored at - 70 C. To prepare the challenge inoculum the isolate was thawed at room temperature, grown overnight in supplemented M96 broth, and inoculated onto supplemented pleuropneumonia-like organisms (PPLO) agar.[28] Inoculated plates were incubated at 37 C for 18-24 hours. Bacteria were harvested in 5.0 ml cold, sterile, phosphate buffered saline solution (PBSS), pH 7.2. The bacterial suspension was centrifuged, the supernatant discarded, and the cells resuspended in 4.0 ml of cold sterile PBSS. The standard plate count of this suspension was 1.4×10^8 colony forming units (CFU) per ml.

Experimental animals - Twenty three cesarean derived colostrum deprived pigs of uniform weight were obtained from a commercial source at five weeks of age. Nasal swabs obtained upon arrival were negative for *H. parasuis*, *Streptococcus suis*, *Bordetella bronchiseptica*, *Pasteurella multocida*, and *Actinobacillus pleuropneumoniae*. Pigs were housed in an isolation facility, fed an 18% protein corn-soybean meal ration, and provided water ad libitum.

Experimental infections - Control and principal pigs were housed in separate isolation rooms. Seventeen principal pigs were inoculated with 0.5 ml of challenge inoculum slowly dripped into each nostril to give a total inoculum dose of 1.4×10^8 CFU. Six control pigs were sham inoculated with sterile PBSS. At 4, 8, 12, 18, and 26 hours post inoculation (HPI) three infected pigs and one control pig were euthanized and necropsied. At 36 HPI, only 2 infected pigs and one control pig were euthanized and necropsied. At necropsy samples were obtained for bacterial culture and light and electron microscopic examination. Specific anatomic locations were used to maintain consistency in sampling the nasal cavity. Samples were obtained from the following locations: the nasal vestibule and rostral 20 percent of the nasal turbinate, a region immediately caudal to the midpoint of the nasal turbinate, and the caudal 20 percent of the nasal turbinate.

Bacteriology - Specimens collected for bacterial culture included blood, lung, tonsil, and swabs from the rostral, middle, and caudal nasal cavity, and trachea. Tubes of supplemented M96 broth and blood agar were inoculated.[28] Blood agar plates were cross streaked with *Staphylococcus aureus*. Broth

cultures were incubated overnight at 37 C and then inoculated on blood agar plates with a *S. aureus* nurse streak. All media was examined for growth after 24 and 48 hours incubation at 37 C. *Haemophilus parasuis* was identified by colony morphology and satellitosis from the *S. aureus* nurse. Representative isolates were submitted to the Iowa State University Veterinary Diagnostic Laboratory for bacterial identification.

Pathology - Tissues from the rostral, middle, and caudal nasal cavity, tonsil, trachea, and lung were fixed in 10% neutral buffered formalin for 24 hours, processed routinely, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin. For electron microscopy, 3 to 6 specimens from each tissue were cut to 1 mm thickness in at least one dimension and placed in McDowell and Trump's 4F:1G fixative (4% formaldehyde, 1% glutaraldehyde) in 0.1 M Sorenson's sodium phosphate buffer.[15] Tissues were rinsed in distilled water, post fixed in 1% osmium tetroxide, rinsed in distilled water, dehydrated in acetones, and embedded in epoxy resins. Semi-thin (1 micron) sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections of selected areas were stained with lead citrate and uranyl acetate and examined on a Hitachi electron microscope.

Immunohistochemistry - Five micron sections from formalin fixed, paraffin embedded blocks were placed on poly L-lysine coated microscope slides, deparaffinized in xylenes, and rehydrated. Endogenous peroxidase activity was quenched by reacting the slides with a 3% hydrogen peroxide solution for 20

minutes. Following washing in Tris buffered saline solution, slides were overlaid with 10% normal goat serum for 20 minutes to prevent background staining. An anti-*H. parasuis* antisera prepared in rabbits was used as the primary antibody at a 1:1000 dilution. Slides were overlaid with primary antibody and incubated in a humidified chamber overnight at 4 C. After washing in Tris-buffered saline, a 1:200 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) adsorbed with 2% normal swine serum was applied to the slides for 30 minutes. Slides were washed with Tris-buffered saline and a 1:200 dilution of peroxidase conjugated streptavidin (Zymed Laboratory Inc., San Francisco, CA) was applied for 30 minutes. Streptavidin was detected with a 0.4% 3-amino-9-ethylcarbazole (AEC) solution. Slides were counterstained with hematoxylin. Negative controls included using normal rabbit serum and Tris buffered saline in place of the anti-*H. parasuis* antisera.

RESULTS

Bacteriologic findings - *Haemophilus parasuis* was consistently isolated from the nasal cavity and trachea (Table 1) and only sporadically isolated from the lung and blood. *Haemophilus parasuis* was not recovered from the tonsil. From sites within the nasal cavity, *H. parasuis* was recovered from the mid-portion of the turbinate of all infected pigs. Cultures from the rostral portion of the turbinate were positive for *H. parasuis* at 4 and 8 HPI while *H. parasuis* was recovered from the caudal nasal cavity at 12, 26, and 36 HPI. *Haemophilus parasuis* was

isolated from the lung at 26 and 36 HPI and from the blood of 1 pig at 36 HPI.

Immunohistochemical findings - *H. parasuis* was sporadically detected in sections of middle and caudal turbinate and trachea (Table 1) and were most frequently detected at 4 and 8 HPI. *Haemophilus parasuis* was not detected in sections of rostral turbinate, tonsil, or lung. Immunoreactivity occurred as patchy focal areas of intense staining at the apical surface of mucosal epithelial cells with minimal background staining. Immunoreactivity was not present within the mucosa or nasal submucosal glands.

Light microscopic findings - Microscopic lesions were consistently present at all time points in sections from the middle and caudal nasal cavity of infected pigs. At 4 HPI, the nasal submucosa was diffusely infiltrated by large numbers of neutrophils. (Figure 1) Neutrophils were present as multifocal intraepithelial aggregates or aggregates accompanied by cell debris within submucosal glands. Lesions present at 8 and 12 HPI were less severe and consisted of moderate numbers of neutrophils within the submucosa and increased numbers of intraepithelial neutrophils. At 26 and 36 HPI, the submucosal infiltrate consisted of decreased numbers of neutrophils compared to early time points and scattered aggregates of lymphocytes. Focal loss of cilia and irregularity of the mucosal epithelium overlying areas of inflammatory cell infiltrate were observed in at least one pig at each time point, but were most consistent at 18 and 26 HPI. (Figure 2) Microscopic lesions of the rostral nasal mucosa were limited to submucosal infiltrates of low numbers of neutrophils in all pigs examined 4 HPI.

Microscopic lesions of the trachea were observed at 12, 18, 26, and 36 HPI. At 12 HPI, there were widely scattered, focal, submucosal infiltrates of neutrophils. This lesion was more prominent at 18 HPI and there were also rare intraepithelial aggregates of neutrophils. By 26 and 36 HPI there were multifocal aggregates of lymphocytes and macrophages, but lesser numbers of neutrophils. Significant lesions were not observed in the tonsil and lung of infected pigs or in tissues from control pigs.

Ultrastructural findings - Ultrastructural changes were limited to the nasal and tracheal mucosa. Lesions were detected in the nasal mucosa of all infected pigs. At 4 and 8 HPI, there were scattered areas in which the apical surface of the mucosa was irregular due to cells which protruded above the surface of adjacent epithelial cells. (Figure 3) These cells lacked cilia and basal bodies and had apical cytoplasm with decreased electron density. In these areas, there was an apparent decrease in the numbers of cilia compared to tissues from control pigs. At all other time points there was more extensive loss of cilia. Affected cells lacked basal bodies and had irregular microvilli. There was mild dilation of the cytocavitary network which was most prominent in the apical portion of the cytoplasm. Multivesicular bodies were prominent in many of these cells. Although there were focal areas of increased intercellular space, tight junctions remained intact. The most severely affected cells were often overlying aggregates of intraepithelial neutrophils. (Figure 4) In addition, there were cells within the

epithelium, interpreted as macrophages, which contained multiple phagolysosomes.

Bacteria were only rarely detected by transmission electron microscopy in sections of nasal mucosa. At 4 HPI low numbers of bacteria were present at the luminal surface of ciliated epithelial cells, often associated with an amorphous, variably electron dense material. At 26 and 36 HPI bacteria were identified between cilia. (Figure 5) Bacterial cells were not in direct contact with cilia and no fimbria-like structures were observed. When bacteria were observed, they were associated with normal epithelial cells adjacent to areas of epithelial cell degeneration.

Ultrastructural changes of the tracheal mucosa were less common and less severe than the changes detected in the nasal mucosa. At 8, 12, 18, and 26 HPI, there was mild cellular protrusion and focal loss of cilia. Significant lesions were not detected at 4 or 36 HPI. Bacteria were rarely detected from samples at 12 and 26 HPI and were present at the apical surface of intact ciliated epithelial cells.

DISCUSSION

There have been few studies on the pathogenesis of *H. parasuis* infection. These studies have focused on the clinical signs and systemic lesions of *H. parasuis* and have not examined mucosal colonization.[5,18,23,25,30] In a recent study utilizing a CDCD pig model of *H. parasuis* infection, *H. parasuis* was

recovered from the nasal cavity, but not from other mucosal sites.[35] That study did not examine multiple sites within the nasal cavity, obtain samples at early post inoculation times, or utilize immunohistochemistry and transmission electron microscopy to characterize the sites of colonization. The present study demonstrated that *H. parasuis* is consistently recovered from the middle and caudal nasal cavity and *H. parasuis* can be demonstrated on the nasal mucosa by immunohistochemistry and transmission electron microscopy. In addition, *H. parasuis* was sporadically associated with the tracheal mucosa. Bacterial cultures and immunohistochemistry failed to demonstrate *H. parasuis* within the tonsil. Other investigators have recovered *H. parasuis* from nasal secretions and identified *H. parasuis* antigens within the tonsil.[4] Failure of the current study to demonstrate tonsilar colonization may be due to the fact an intranasal, rather than oral, route of inoculation was used. However, intranasal inoculation of gnotobiotic pigs with *P. multocida* resulted in colonization of the nasal cavity and the tonsil, with the tonsil being colonized in higher numbers as determined by quantitative bacterial culture.[1] The tonsil is an important site of colonization for *S. suis* and *P. multocida* in swine.[1,2,36] In contrast, *H. parasuis* may preferentially colonize the nasal mucosa. This is consistent with culture results from slaughter swine in which *H. parasuis* was frequently isolated from the nasal cavity but was recovered from the surface or cut surface of the tonsil in only one case.[17] The present study did not use quantitative bacterial cultures to determine the magnitude of bacterial growth at mucosal sites. Further studies using oral and intranasal routes of

inoculation, different *H. parasuis* strains, and quantitative bacterial cultures may aid in determining the relative importance of the tonsil as a site of colonization of *H. parasuis*.

Haemophilus parasuis is often isolated from the nasal cavity of healthy pigs and from pigs with rhinitis.[10,27] In the present study *H. parasuis* was only transiently recovered from the rostral portion of the nasal cavity. This portion of the nasal cavity is covered by a stratified squamous epithelium with a gradual transition to the ciliated pseudostratified columnar epithelium of the middle to caudal nasal turbinates.[3] This type of epithelium may be more resistant to colonization. *Haemophilus parasuis* was consistently isolated from the middle portion of the nasal cavity and induced an acute purulent rhinitis. Colonization of *P. multocida* in gnotobiotic pigs was not associated with lesions of the respiratory mucosa; however, in that study bacterial numbers in the inoculum were much lower than the dose of 10^8 CFU used in this experiment. The severity and extent of the mucosal lesion in this study may be influenced by the high number of bacteria in the inoculum.

In addition to producing a purulent rhinitis, *H. parasuis* infection produced focal loss of cilia and acute cell swelling within the nasal and tracheal mucosa as demonstrated by light and electron microscopy. These changes were not seen in control samples obtained from similar sites. Lesions were most severe in areas adjacent to or overlying significant infiltrates of neutrophils. These alterations to the mucosal surface may alter defense mechanisms and allow *H. parasuis* to

invade the mucosa and gain access to the blood stream. Bacteria were not detected in association with the areas of cellular degeneration, however macrophages within the epithelium contained multiple phagolysosomes indicating phagocytic activity. The failure to detect bacteria associated with the areas of cilia loss and cellular degeneration may be due to the small sample size examined or bacteria on the surface may have been lost during processing. An alternate explanation is that *H. parasuis* may initially associate with the mucus layer and produce a soluble toxin which causes the cellular changes. *Haemophilus influenzae* lipopolysaccharide and an *H. influenzae* derived low molecular weight substance may produce similar changes in the pathogenesis of *H. influenzae* infections in humans.[20,21]

Haemophilus influenzae has been shown to colonize a mucous layer and nonciliated cells, does not colonize intact ciliated epithelial cells, and induces ciliostasis, loss of cilia and epithelial cell degeneration in human nasopharyngeal tissue.[9,29,33] *Haemophilus influenzae* has also been shown to break down epithelial cell tight junctions, invade between cells, and pass into the submucosa.[33] Based on the evidence presented in this study we hypothesize that *H. parasuis*, like *H. influenzae*, initially associates with the mucus layer of the nasal cavity and damages the mucosal epithelium and induces inflammation by release of one or more toxic compounds. This cellular damage compromises the mucosal barrier and may lead to invasion and systemic spread of the infection.

This study did not determine a specific cell type or structure colonized by *H. parasuis*. Bacterial cells were rarely seen by transmission electron microscopy. When present, bacterial cells were not closely apposed to cilia or other cell structures. This is in contrast to *Mycoplasma hyopneumonia* and *Bordetella bronchiseptica* which colonize ciliated epithelial cells in high numbers and are often intimately associated with the cilia.[7,8,16,37]

Mechanisms of colonization of *H. parasuis* remain poorly defined and attachment appendages were not seen in this study. Fimbriae of *H. parasuis* have been observed on bacteria inoculated on chicken chorioallantoic membranes, but not on cells cultivated *in vitro*.[22] Fimbriae can be important attachment structures in bacterial pathogenesis and have been demonstrated in Streptococcus suis[13] and P. multocida[6] from swine. Capsular material may be important in attachment to mucosal surfaces.[12] Initial reports suggested that virulent strains of *H. parasuis* did not possess a capsule.[19] In a study using a guinea pig model of *H. parasuis* infection, encapsulation of a virulent *H. parasuis* strain was demonstrated by capsular staining.[28] The isolate used in this study possessed a capsule as determined by Maneval staining.[14] (J. Vahle, personal observation) Further efforts to determine the presence of fimbria or capsular material may aid in identifying virulence factors of *H. parasuis*.

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Fig. 1. Nasal turbinate; CDCD pig 4 hours post *H. parasuis* infection. Note submucosal and intraepithelial infiltrates of neutrophils and aggregates of neutrophils and cellular debris within the lumen of a nasal submucosal gland (arrow). HE. Bar = 20 microns.

Fig. 2. Nasal turbinate; CDCD pig 18 hours post *H. parasuis* infection. Note mild submucosal and intraepithelial infiltrates of lymphocytes. There are decreased number of cilia present. HE. Bar = 20 microns.

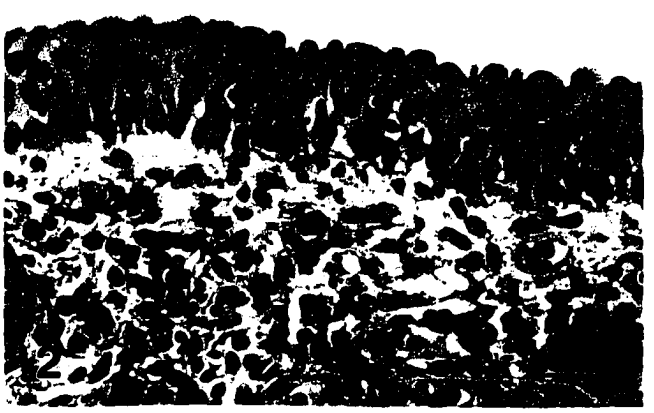

Fig. 3. Nasal mucosa; CDCD pig 4 hours post inoculation. There is multifocal cell protrusion. (arrows) There are decreased numbers of cilia. A cell containing phagolysosomes within the mucosa is identified as a macrophage. (m) Bar = 1 micron.

Figure 4. Nasal mucosa; CDCD pig 36 hours post inoculation. An intraepithelial neutrophil (n) is present. There is diffuse loss of cilia and basal bodies, microvilli are irregular and reduced in size, and there is dilation of the cytocavitary network within the apical cytoplasm. Bar = 1 micron

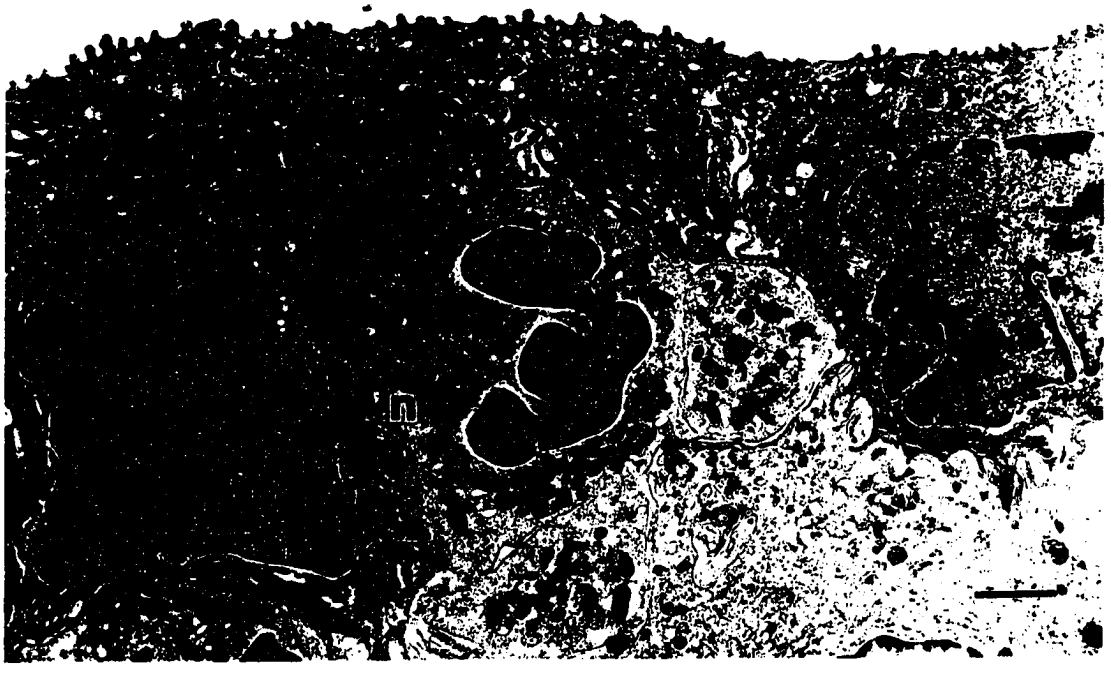

Figure 5. Nasal mucosa; CDCD pig 26 hours post inoculation. Rare bacteria (arrows) are present between cilia. Bacteria are not closely apposed to cilia. Bar = 0.5 micron.

Table 1. Summary of Bacteriologic and Immunohistochemical Findings in Pigs Inoculated with *H. parasuis*.

HPI	Rostral Nasal		Mid. Nasal		Caudal Nasal		Tonsil		Trachea		Lung		Blood
	BACT*	IHC†	BACT	IHC	BACT	IHC	BACT	IHC	BACT	IHC	BACT	IHC	BACT
4	3/3	0/3	3/3	1/3	1/3	2/3	0/3	0/0	2/3	2/3	0/3	0/0	0/3
8	3/3	0/3	2/3	2/3	0/3	1/3	0/3	0/0	2/3	2/3	0/3	0/0	0/3
12	1/3	0/3	3/3	1/3	2/3	0/3	0/3	0/0	2/3	1/3	0/3	0/0	0/3
18	1/3	0/3	3/3	1/3	1/3	1/3	0/3	0/0	3/3	1/3	0/3	0/0	0/3
26	1/3	0/3	3/3	0/3	2/3	0/3	0/3	0/0	3/3	2/3	2/3	0/0	0/3
36	0/2	0/2	2/2	0/2	2/2	0/2	0/2	0/0	1/2	0/3	1/2	0/0	1/2

*Bacterial Culture: Number culture positive for *H. parasuis*/number sampled

†Immunohistochemistry: Number *H. parasuis* antigen positive/number sampled

CHAPTER 5: INTERACTION OF *HAEMOPHILUS PARASUIS* WITH SWINE NASAL TURBINATE EXPLANTS

A paper to be submitted to Veterinary Pathology

J.L. Vahle, J.S. Haynes, and J.J. Andrews

ABSTRACT. *Haemophilus parasuis* is a common cause of polyserositis and polyarthritis in young swine. *Haemophilus parasuis* is often isolated from the nasal cavity of swine and the nasal mucosa appears to be an initial site of colonization. In this study a swine nasal mucosal explant system was used to investigate the effects of *H. parasuis* on the nasal mucosa of swine. Forty six viable explants were obtained from 3 to 4 week old specific pathogen free swine. Nasal turbinate explants were embedded in agarose and maintained in cell culture media. In this system explants maintained ciliary vigor for up to 72 hours post harvest. Twenty seven principal explants were infected with 2 to 4 x 10^8 CFU of *H. parasuis* per ml of cell culture media and 19 control explants were maintained in non-infected cell culture media. Ciliary vigor was evaluated and bacterial cultures were performed on all explants. Transmission electron microscopy was performed on 9 infected and 7 control explants. Control explants maintained ciliary activity and had normal morphology. Infected explants had a marked reduction in ciliary activity and ultrastructural changes of cell protrusion, cell

blebbing, and cilia loss. These changes suggest *H. parasuis* infection of swine nasal mucosa results in ciliostasis and damage to mucosal epithelial cells.

INTRODUCTION

Colonization of a mucosal surface is an important step in the pathogenesis of many bacterial infections. Colonization allows for the establishment of infection, provides a source for transmission to other individuals, and serves as a possible site for mucosal invasion preceding systemic disease.[1,3,16] Determining the mechanisms of colonization is important in understanding one of the steps in the pathogenesis of bacterial infections of mucosal surfaces.

Haemophilus parasuis is a common cause of polyserositis and polyarthritis in growing swine.[12] Although the disease was first described by Glasser in 1910,[7] little attention has been paid to determining the sites and mechanisms of mucosal colonization. We utilized experimental inoculation of cesarean derived, colostrum deprived (CDCD) pigs to study *H. parasuis* infection.[18] (J. Vahle, manuscript submitted for publication) In these studies, *H. parasuis* was consistently recovered from the middle and caudal nasal cavity and trachea and *H. parasuis* antigens were detected on the nasal mucosa. In addition, *H. parasuis* infection resulted in a mild to moderate suppurative rhinitis. Ultrastructural changes included acute cell swelling and decreased numbers of cilia. Bacteria were rarely visualized.

An *in vitro* system is useful in evaluating the interaction of bacteria with a mucosal surface or individual mucosal epithelial cells. Human pathogens such as *Haemophilus influenzae* and *Neisseria meningitidis* have been studied using nasopharyngeal mucosal explants.[16] Colonization by swine mucosal pathogens *Pasteurella multocida*, *Bordetella bronchiseptica*, and *Streptococcus suis* have been studied using isolated epithelial cells, tracheal ring explants, nasal turbinate fragments, or lung slices.[2,4,5,8,9,13] Models which utilize intact mucosa are useful as ciliary activity can be evaluated by the use of phase contrast microscopy.

Haemophilus influenzae is an important cause of meningitis and can also cause otitis, sinusitis, epiglottitis, and pneumonia in humans.[15,16] Mucosal colonization by *H. influenzae* has been investigated using a nasopharyngeal explant system. In this model, *H. influenzae* has been shown to colonize a mucous layer and nonciliated cells, to induce ciliostasis, and to cause loss of cilia and epithelial cell degeneration.[6,15,16] *H. influenzae* is thought to invade the mucosa and pass into the submucosa by passing between disrupted epithelial cells.[16]

Based on observations that *H. parasuis* is present within the nasal cavity, produces epithelial cell degeneration and inflammation of the nasal mucosa, and has not been demonstrated to colonize a specific cell type, we hypothesize that *H. parasuis*, similar to *H. influenzae*, initially associates with a mucous layer within the nasal cavity and induces ciliostasis, loss of cilia, and epithelial cell degeneration. These changes may lead to mucosal invasion and systemic

infection. The goal of this study was to develop a swine nasal mucosal explant system and use that system to evaluate the interaction of *H. parasuis* with nasal mucosa *in vitro*.

MATERIALS AND METHODS

Bacterial inoculum. An isolate of *H. parasuis,* designated HPS 5, from a field case of polyserositis was thawed at room temperature, grown overnight in supplemented M96 broth, and inoculated onto supplemented pleuropneumonia-like organisms (PPLO) agar.[14] PPLO plates were incubated at 37 C for 24 hours. Bacteria were harvested by washing each plate with 5.0 ml of cold, sterile phosphate buffered saline solution (PBSS). Bacteria were washed once in PBSS, harvested by centrifugation, and resuspended in 2.5 mls of PBSS.

Nasal explants. Nasal turbinates were obtained from 3 to 4 week old specific pathogen free pigs. The nasal turbinates from each pig were placed in a 50 mls of minimal essential media (MEM) containing Earle's salts, L-glutamine, and sodium bicarbonate (Sigma Chemical Company, St. Louis, MO) and supplemented with 20 % vol/vol fetal bovine sera (FBS)(Atlanta Biologicals, Norcross, GA), penicillin (100 units/ml), streptomycin sulfate (0.1 mg/ml) and gentamicin sulfate (0.05 mg/ml)(Sigma Chemical Company, St. Louis, MO). Turbinates were evaluated for ciliary motility using phase contrast inverted microscopy. Turbinates which did not demonstrate vigorous ciliary motility were

discarded. Fragments 4 to 8 mm square were obtained from the middle and caudal portions of each suitable turbinate. These fragments were placed in fresh MEM containing antibiotics and incubated at 37 C for 3-4 hours to allow recovery from trauma. Fragments were transferred to fresh MEM without antibiotics and incubated for 1 to 2 hours. The fragments were then placed on 1.5 % molten agarose which covered the bottom of a 4 cm diameter petri dish using aseptic technique in a laminar flow hood. As the molten agar solidified, additional molten agar was pipetted around the edge of the explant to ensure that only the mucosal surface was exposed. The explant was covered with MEM without antibiotics and ciliary activity was evaluated. Explants which lacked vigorous ciliary motility were discarded.

From each pig approximately 8-15 viable explants were obtained. A total of 46 viable explants were obtained from 4 pigs. Of the viable explants, 27 were infected and 19 were used as control explants. Explants were infected by replacing the media with 6.5 mls of MEM without antibiotics and 0.5 ml of the bacterial inoculum. For control explants, the media was replaced with 7.0 mls of MEM without antibiotics. The standard plate count of the infected media at the time of inoculation was between 2 and 5 x 10^8 CFU/ml. In addition to the explants, petri dishes containing media alone and media with *H. parasuis* were used as controls to monitor sterility.

Ciliary activity for each explant was monitored subjectively at 2 and 4 hours post inoculation (HPI). Ciliary activity was visualized at the edge of the

explant and was given a subjective score (0=no motion, 1=weak ciliary motion, 2=vigorous ciliary motion). Explants were also observed for the accumulation of mucous on the surface of the explant. In initial replicates, control explants were retained for up to 72 HPI.

Media from two infected explants from each pig and from petri dishes which lacked explants was sampled at 2 and 4 HPI for quantitative bacterial culture. At 4 HPI, media from all explants were cultured. The explants were then divided into samples for direct bacterial culture and light microscopy. Sections from 9 infected explants and 7 control explants were placed in 3 % glutaraldehyde in 0.1 M sodium cacodylate buffer for transmission electron microscopy. The remainder of the explant was placed in 10% neutral buffered formalin for light microscopy. Following fixation, samples for electron microscopy were rinsed in distilled water, post fixed in 1 % osmium tetroxide, rinsed in distilled water, dehydrated in acetones, and embedded in epoxy resins. Semi thin, toluidine blue stained sections were examined by light microscopy. Ultrathin sections were made from selected areas, stained with lead citrate and uranyl acetate, and examined on a Hitachi electron microscope. Tissues for light microscopy remained in fixative for 24 hours, were processed routinely, embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin.

RESULTS

Ciliary vigor remained strong in control explants at both 2 (average score = 2) and 4 (average score = 1.9) hours post inoculation. In addition, the surface of control explants remained clear or was covered by scant amounts of mucus. In initial experiments, control explants were maintained in culture for up to 72 hours post inoculation with no visible decrease in ciliary vigor. In contrast, the ciliary vigor of infected explants was mildly decreased at 2 hours post inoculation (average score = 1.6) and markedly decreased at 4 hours post inoculation (average score = 0.9) with 9 of 27 infected explants having no visible motility. In explants which lacked visible ciliary motion, the surface of the explant contained mild to moderate amounts of mucus.

Bacteria were not recovered from the media of control explants. Standard plate counts of media from selected infected explants remained between 2 and 4 x 10^8 CFU per milliliter at 2 and 4 HPI. Pure cultures of *H. parasuis* were obtained from the surface of infected explants at 4 HPI. *H. parasuis* was recovered from media which contained bacterial inocula but no explants. Standard plate counts from these samples remained between 3 to 4.8 x 10^8 CFU/ml.

Examination of control explants by light microscopy revealed normal morphology. The mucosal surface contained a uniform population of cilia and mucosal epithelial cells were intact. (Figure 1) In a few control explants there was patchy degeneration and necrosis of submucosal glands. Epithelial cells within

these glands had pyknotic nuclei and disrupted cytoplasmic borders. Light microscopy of infected explants revealed minimal changes. In the most severely affected areas there was patchy loss of cilia, cilia occasionally appeared clumped, and the apical surface of the epithelial cells protruded above adjacent epithelial cells. (Figure 2) In areas, detached, degenerate epithelial cells were present above the mucosal surface.

Ultrastructural examination of control explants revealed a uniform apical mucosal surface, intact cilia and basal bodies, and intact intercellular junctions. (Figure 3) Cell protrusion or cell blebbing was not present, however, there was mild to moderate mitochondrial swelling of scattered mucosal epithelial cells. In infected explants there were multifocal areas in which mucosal epithelial cells protruded above the adjacent epithelial cells. (Figure 4) In these protruded cells, there was decreased electron density and lack of organelles in the apical cytoplasm. In addition, these areas had apparently decreased numbers of cilia and basal bodies compared to control explants. Scattered cells contained variably sized fragments of cytoplasm blebbing from the apical mucosal surface. (Figure 5) These structures typically lacked cell organelles, but occasionally contained basal bodies. Many of these cells had mitochondria which were swollen and had decreased electron density, however cristae remained intact. In rare cells, increased numbers of multivesicular bodies were present and there was dilation of the cytocavitary network. In severely affected areas there were scattered necrotic mucosal epithelial cells which were contracted, lacked organelles, and were

diffusely electron dense. With the exception of cells adjacent to necrotic cells, tight junctions remained intact. Bacteria were seen frequently between cilia, often in small clusters. (Figure 6) The bacteria were not intimately associated with cilia or the apical mucosal surface. Structural appendages between the bacteria and cilia were not identified.

DISCUSSION

A variety of *in vitro* systems have been developed to study the adherence of bacteria to swine respiratory mucosa.[2,4,9,13] In a study investigating *Bordetella bronchiseptica* and *Pasteurella multocida*, swine nasal turbinate fragments were determined to be a more suitable system for evaluating bacterial adherence than isolated swine nasal epithelial cells.[2] In that study incubation longer than 3 hours resulted in loss of cilia, however, effects on ciliary activity were not evaluated. A human ciliated nasopharyngeal explant system has been used to investigate the pathogenesis of *Neisseria meningitidis* and *Haemophilus influenzae*.[15,17] Both of these models allow evaluation of ciliary activity. In one of these models, portions of respiratory mucosa are embedded in agar to prevent bacteria from colonizing and invading the submucosa or basolateral portions of epithelial cells. In this study, we adapted these techniques to a swine nasal turbinate explant model. This system has advantages over previous swine models in that the mucosal surface remains intact, bacteria are not directly exposed to the submucosa, and ciliary function can be evaluated. Disadvantages of the system described in this

study are the low number of explants available from each pig and the need to use nasal turbinates from high health status pigs. In preliminary studies, explants from conventional pigs which had subclinical rhinitis had very poor viability. Although not done in this study, the system could be used to quantify adherence, investigate specific adhesions molecules, and evaluate compounds which block adherence.

In this study, the swine nasal turbinate explant system was used to investigate the effects of *H. parasuis* on ciliary activity at 2 and 4 HPI and on the morphology of the explants at 4 HPI. *Haemophilus parasuis* infection of the explants produced marked reduction in ciliary activity as determined by qualitative observation with phase contrast inverted microscopy. In addition, there was ultrastructural damage to the ciliated epithelial cells without direct colonization of the cilia or apical mucosal surface. *Haemophilus influenzae* Type b has been shown to induce ciliostasis without directly coionizing the ciliated epithelial cells.[6] Ciliostasis may be mediated by either lipopolysaccharide or a low-molecular weight substance.[10,11] *Haemophilus parasuis* may utilize a similar mechanism to induce ciliostasis as bacteria were not intimately associated with cilia in high numbers. Additional studies on the effects of cell free culture supernatant and killed bacterial suspensions on the nasal explants will be needed to determine the role of extracellular products of *H. parasuis* in producing ciliostasis.

The morphologic features of *H. parasuis* infection of nasal turbinate explants are similar to those seen in infection of CDCD swine and to the early

stages of *H. influenzae* type b infection of nasopharyngeal explants. These changes include cell protrusion, loss of cilia, and mitochondrial swelling.[15,149] In addition to these lesions, *H. influenzae* type b has been shown to produce breakdown of intercellular tight junctions, intercellular invasion of the mucosal surface, and passage into the submucosa.[16] These changes were not observed in this study. In this study, explants were only sampled for microscopy at 4 HPI. Additional studies using longer incubation periods will be useful in evaluating subsequent events in mucosal colonization and invasion of *H. parasuis.*

This study demonstrated that swine nasal mucosal explants remain viable in an agar embeddment model and this may be a useful system to study mucosal colonization of swine pathogens. *H. parasuis* infection of explants resulted in ciliostasis and in early lesions of acute cell swelling and ciliary loss. We hypothesize that these alterations to the mucosal surface facilitate mucosal invasion by *H. parasuis* and allow access to submucosal blood vessels resulting in bacteremia. In addition, mucosal injury produced by *H. parasuis* may enhance the colonization and invasion of other bacterial pathogens of the nasal mucosa of swine.

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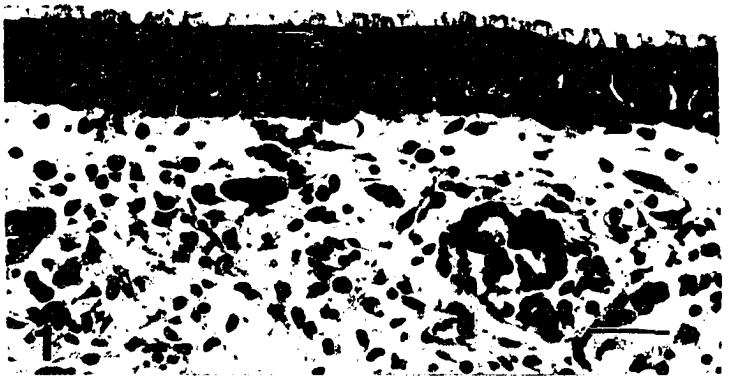
Figure 1. Nasal mucosal explant; Non-infected control. Note the regular apical mucosal surface and even distribution of cilia. HE. Bar = 25 microns.

Figure 2. Nasal mucosal explant; 4 hours post inoculation with *H. parasuis*. The apical mucosal surface is irregular, there is focal loss of cilia, and mild cell protrusion. Detached degenerate epithelial cells are present (arrow). HE. Bar = 25 microns.



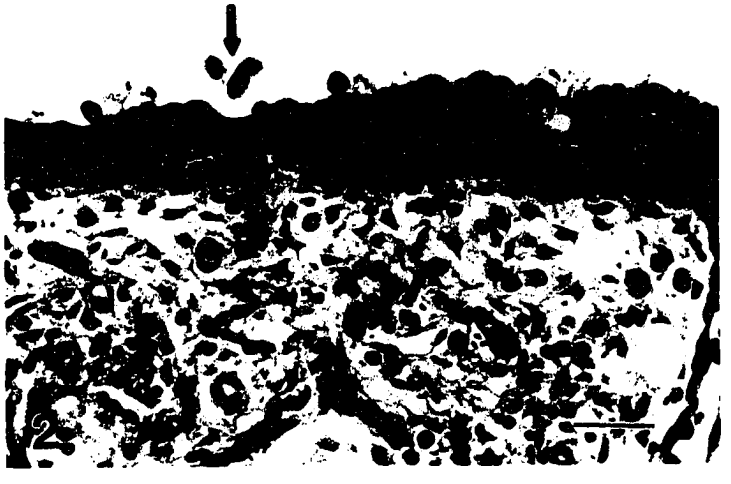


Figure 3. Nasal mucosal explant; non-infected control. The apical mucosal surface is regular, cilia are evenly distributed, and mitochondria are intact. Bar = 2 microns.

Figure 4. Nasal mucosal explant; 4 hours post inoculation with *H. parasuis*. There is focal mucosal epithelial cell protrusion, reduced numbers of cilia, attenuation of microvilli, and swelling of mitochondria. Bar = 2 microns.

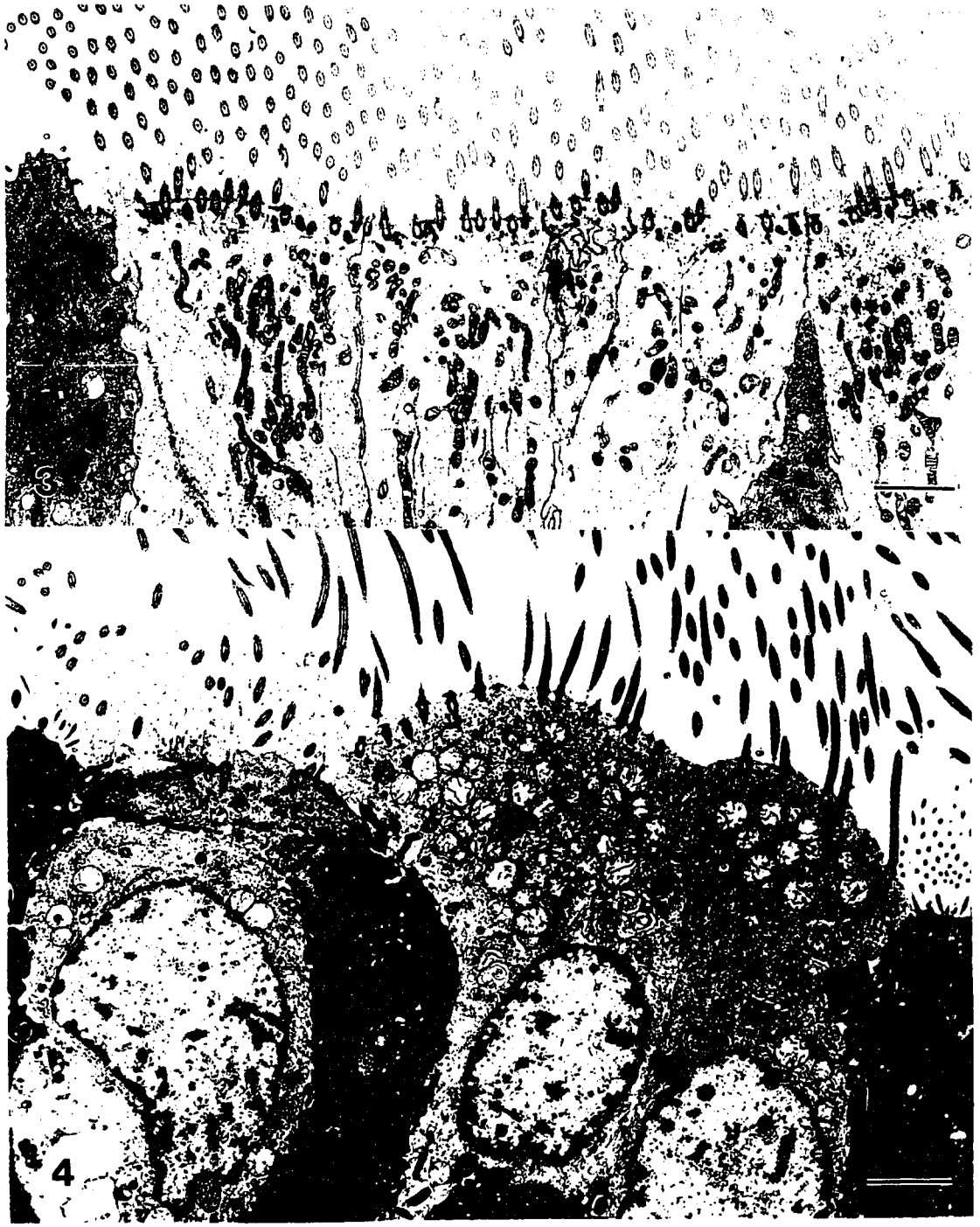

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Figure 5. Nasal mucosal explant; 4 hours post inoculation with *H. parasuis*. There is blebbing at the apical surface of a ciliated epithelial cell (arrow). An adjacent epithelial cell has increased electron density. Bar = 2 microns.


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Figure 6. Nasal mucosal explant; 4 hours post inoculation with *H. parasuis.* Bacteria are present between cilia and near the apical mucosal surface. The subjacent epithelial cell is protruding, however tight junctions remain intact (arrow). Bar = 2 microns.

CHAPTER 6: GENERAL CONCLUSIONS

Although there have been several studies reporting experimental infection of swine with *Haemophilus parasuis*, little attention has been given to describing the sequence of events which follow aerosol exposure of naive swine. The goals of this work were to develop an experimental model of *H. parasuis* infection in swine and use that model to characterize the clinical, bacteriologic, and morphologic findings in a temporal manner. In addition, this work sought to examine the mucosal colonization of *H. parasuis* using bacteriologic, light microscopic, and ultrastructural methods.

The first manuscript describes an experimental model of *H. parasuis* infection in cesarean derived, colostrum deprived (CDCD) swine. The model uses an intranasal route of inoculation and pigs were examined at various time points post inoculation. In this model clinical findings were typical of field cases of *H. parasuis* infection and included pyrexia, lethargy, lameness, and inappetence. Gross lesions of polyserositis and polyarthritis were also typical of field cases. Microscopic findings consisted of fibrinopurulent inflammation at serosal surfaces and a mild suppurative rhinitis. These results suggest that *H. parasuis* infection of naive swine results in polyserositis and polyarthritis without significant lesions of pneumonia. There are reports that *H. parasuis* can produce a bronchopneumonia, however these reports are either from experimental infections with a high volume inocula or from field cases in which there are other respiratory

pathogens present.[4,13,14] This study is the first to characterize the bacteriologic findings at mucosal and systemic sites in a temporal manner. *Haemophilus parasuis* was initially recovered from the nasal cavity and trachea and not recovered from the tonsil. Following recovery from mucosal sites, *H. parasuis* was recovered from the blood and a variety of serosal sites.

The initial study provided preliminary evidence that *H. parasuis* initially associated with the nasal mucosa, gained access to the blood stream, and replicated and induced lesions at serosal surfaces. Other investigators have reported that *H. parasuis* resides in the nasal cavity.[7,9,14] As mucosal colonization is an important step in the pathogenesis of many bacterial infections, we sought to more fully define the sites and mechanisms of mucosal colonization by *H. parasuis*. Using the previously developed experimental model, the second study sought to determine the sites of mucosal colonization, evaluate morphologic changes associated with colonization, and determine if a specific cell type or cell structure was colonized. In this study *H. parasuis* was identified on the mucosa of the middle and caudal nasal cavity and the trachea by bacterial culture, immunohistochemistry, and electron microscopy. The presence of bacteria was associated with a mild to moderate suppurative rhinitis. Ultrastructural changes of the mucosal surface included cell protrusion and loss of cilia. Bacteria were not frequently seen by electron microscopy, but when present they were in low numbers within a mucous like layer or located between cilia. These findings support previous observations that the nasal mucosa is the initial site of

replication. In addition, this study failed to demonstrate colonization of the tonsil. In one report of experimental infection of swine with *H. parasuis*, antigens of *H. parasuis* were detected in the tonsil but bacterial cultures were negative.[3] The tonsil is an important site of colonization for *Pasteurella multocida* and *Streptococcus suis*.[1,2,20] This study also suggests that *H. parasuis* does not heavily colonize a specific cell type or structure but does produce mucosal inflammation, cellular degeneration, and cilia loss. These changes likely predispose the mucosal surface to bacterial invasion. *Haemophilus influenzae* Type B (Hib) has been shown to produce similar mucosal lesions.[17,18]

The third component of the dissertation sought to develop a nasal explant system to further evaluate functional and morphologic features of *H. parasuis* infection of nasal mucosa. A variety of *in vitro* systems have been used to evaluate the interaction of bacteria with a mucosal surface including oropharyngeal explants, nasal mucosal explants, tracheal rings, and isolated epithelial cells.[5,6,8,12,15,18,19] In this study we developed a swine nasal turbinate explant system. Turbinates were harvested from young swine, washed in antibiotic containing media, and maintained in antibiotic free cell culture media. The explants were embedded in agarose so that only the mucosal surface was exposed to the media. The explants were examined for ciliary activity by phase contrast microscopy. Uninfected explants maintained fair to excellent ciliary motility for up to 72 hours post inoculation. Infected explants had decreased ciliary motility and increased accumulation of mucus on the mucosal surface. By

light microscopy there were mild degenerative changes of the mucosal surface of infected explants. Ultrastructural lesions in infected explants were similar to those seen in the nasal turbinates of experimentally infected swine and included cell protrusion and patchy loss of cilia. Bacteria did not heavily colonize either cilia or the cell surface of damaged epithelial cells. The ciliostasis and ultrastructural lesions seen in this system are similar to those described as early lesions in the infection of human oropharyngeal mucosa with *H. influenzae* Type b. These findings support the observations made in the previous study using CDCD swine and also suggest that *H. parasuis* infection of a mucosal surface may produce ciliostasis.

Based on the observation of these studies a general pathogenesis for *H. parasuis* infection can be proposed. Following exposure of naive swine, *H. parasuis* associates with a mucous layer in the nasal cavity and produces functional and structural damage to the nasal mucosa including ciliostasis, loss of cilia, and epithelial degeneration. We hypothesize that *H. parasuis* then colonizes and invades the damaged mucosa, gains access to the blood stream, replicates at serosal surfaces, and induces fibrinopurulent inflammation at these systemic sites. In addition, we suggest that the initial mucosal damage is mediated by an unidentified bacterial toxin. This is based on the fact that mucosal damage is present without direct bacterial colonization. A similar pathogenesis is suggested for *Haemophilus influenzae* and may be mediated by either lipopolysaccharide (LPS) or a low molecular weight substance.[10,11,16,17]

In summary, this work characterizes some of the events in the pathogenesis of *H. parasuis* infection in naive swine. This work should provide a background for additional investigations to define the mechanisms of colonization and invasion. Determining these mechanisms will further our understanding of the this disease and may lead to identification of virulence attributes of *H. parasuis*. These virulence attributes may then be targets of strategies to prevent disease caused by *H. parasuis* infection. Other facets of the pathogenesis of *H. parasuis* infection which should be further evaluated include mechanisms of survival in the blood stream, predilection for serosal surfaces, and the role of extracellular toxins in inducing lesions at systemic sites. In addition, there is a need for basic information in the areas of epidemiology, immunology, and bacterial genetics. It is hoped that this work will assist investigators in these areas obtain a general understanding of the pathogenesis of *H. parasuis* infection in swine.

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